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14. ABSTRACT

ALS remains a devastating neurodegenerative disease with no curative treatments available. It is becoming increasingly clear that the accumulation of misfolded and aggregated proteins underlie the pathologies of several major forms of ALS. Protein products of ALS genes, including SOD1 and TDP-43, have been involved in the pathology of protein aggregation. In the past year of research, we have made significant progress towards identifying novel therapeutic agents against protein aggregation and delivering the agents to the relevant nervous systems. Our work has revealed that inhibitors of specific targets have robust effects on protein aggregation in a protein-dependent manner. We have also demonstrated significant progress in delivering agents to in vivo animal models. Further studies are required to pursue some of the new findings that were observed.

15. SUBJECT TERMS
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Introduction

The studies in my laboratory have been directed toward understanding the basic mechanism of ALS, with the goal of unlocking the mysteries of this motor neuron disease, for which effective therapy is lacking. In the past 17 years, our studies have contributed to the realization that protein misfolding plays a critical role in the pathogenesis of ALS. The work of myself and others contributed to the recognition that protein misfolding and aggregation are a unifying feature of disease-causing SOD1 mutants in ALS. Our recent work reveals a previously unrecognized regulation of protein quality control system imparted by the RNA-binding proteins TDP-43, from *C. elegans* to mammals (Zhang et. al., JBC 2012 and unpublished data). Most recently, we discovered a mechanism that explains the nucleotide structural basis at the root of the *C9orf72*-linked ALS/FTD pathogenesis (Haeusler et. al., Nature 2014). Again, the aberrant *C9orf72* repeat RNAs and their binding-proteins cause proteotoxic stress such as unconventionally translated misfolding peptides from the repeat RNAs.

In this project, we hypothesized that the cellular defense system can be harnessed to treat ALS by reprogramming the cell's protein quality control systems. Towards this end, we aim to explore two candidate pathways for reprograming quality control systems in experimental settings. We are taking advantage of the innovative approach of combining *C. elegans* and mammalian approaches. Our approach is to first evaluate and dissect the mechanisms of anti-proteotoxicity candidates in easily tractable *C. elegans* and mammalian cell models. New targets could be identified by studying the mechanisms related disease genes and their modifiers. Then the validated candidates will be explored for *in vivo* targeting in mouse models. Potential therapeutic agents such as shRNAs against target genes will be developed and characterized. The delivery approaches of the novel agents will be explored as well. Together, we hope our efforts will ultimately lead to a wide-spectrum anti-proteotoxicity strategy that could have a broad impact on the treatment of both familial and sporadic forms of ALS.

We would like to thank the program officer for the insightful feedback on our original Year 1 progress report. We have made multiple revisions in the rewritten progress report based on the suggestions. **First**, we have added significantly more comprehensive description of our results supporting the hypothesis/conclusion that TDP-43 and SOD1 represents opposing activities on protein quality control (see new Figures 3-4 and the related description in Result D). **Next**, we propose to explore the novel function of TDP-43 in protein quality control in light of the recent findings. As suggested by the program officer, we have revised the SOW accordingly (see Task 2 last sentence) and attached it with this progress report. **Finally**, in the Tasks 3-4 for Aim 2, we propose to study the newly identified downstream effector, p53, shared between suppressors UBE4B and KDM1A. In Task 4, we plan to determine whether targeting the common effector p53 in the SUNS pathway would have an effect in alleviating disease in ALS mouse models. This plan represents an improved approach to activate the SUNS pathway on a single target using existing drugs. We have revised the SOW accordingly and the justifications are provided as new results shown in the progress report (see new Figures 9 and 10). Also, the **revised SOW** and an **IACUC-approved form** of changes in procedure are in the appendices in this progress report.

Body

Aim 1: To determine whether HSP90 inhibitors attenuate disease phenotypes in *C. elegans* and mouse models of ALS.

Overview:

In this Aim, we hypothesize that increased HSF-1 activity is broadly protective for ALS. In a genome-wide screen for regulators of SOD1 protein aggregation and related disease symptoms, the largest group of identified genes encoded proteins involved in quality control, with the strongest being the master transcription factor HSF1 (Wang et al., 2009a; Wang et al., 2009b). In a related finding, a reduction in HSF-1 activity dramatically precipitated neuronal toxicity in TDP-43 transgenic C. elegans models of ALS (Zhang et al., 2011). HSP90 forms a protein complex with HSF1, and it has been shown that inhibition of HSP90 releases HSF1 to be activated (Zou et al., 1998).

Surprisingly, our recent research in this Aim suggests that HSP90 has opposing effects on the protein levels and aggregation of SOD1 and TDP-43 (see Results B & C below). However, this result is consistent with an emerging notion that SOD1 and TDP-43 paly very different roles in protein quality control as related to the disease pathogenesis, as suggested our work and others. For example, enhancing the phosphorylation of protein translation elongation factor eIF2a has a protective effect on the disease development in SOD1-ALS mouse models (Wang et al., 2014); however, it is the inhibition of the phosphorylation of eIF2a that alleviates the toxicity of TDP-43 in disease cell and fly models (Kim et al., 2014). We believe that the difference between SOD1 and TDP-43 can be explained by a previously unrecognized role of TDP-43 in regulating protein quality control that we recently uncovered (see Result D below). In light of these recent findings, we will explore the mechanisms of interactions between HSP90 and TDP-43, while continuing evaluating the effects of HSP90 inhibitors on SOD1-ALS mouse models.

Task 1: Test the therapeutic effects of the HSP90 inhibitors on SOD1/TDP-43-induced toxicity in both *C. elegans* and mammalian cell models of amyotrophic lateral sclerosis (ALS).

Task 2: Examine the protective effects of NVP-BEP800 treatment in mouse models of ALS.

Results and Findings:

A. The mammalian HSP90 inhibitor NVP-BEP800 does not have a significant effect on the proteotoxicity in *C. elegans* models of ALS.

NVP-BEP800 is a potent and selective inhibitor that binds to the N-terminal ATP-binding pocket of mammalian HSP90 (Massey et al., 2010). To determine the effect of NVP-BEP800 in ALS models of simple organisms, we employed our *C. elegans* models of SOD1(Wang et al., 2009a) and TDP-43(Zhang et al., 2011) that we have established by expressing the human proteins in the nervous system. These *C. elegans* models develop pronounced ALS-like phenotypes, including locomotor defects. As in mammals, movement in *C. elegans* is mediated by muscles that receive signals from motor neurons. The locomotor phenotype is readily measurable and serves as a direct readout of neuronal function.

The N_2 Bristol *C. elegans* wild-type and transgenic strains were cultured on NGM agar plates under standard conditions at 20 ^{o}C (Brenner, 1974). The agar plates were prepared with 0, 50nM, 5nM, or 0.5 nM NVP-BEP800, stored at 4 ^{o}C before experiments, and used within two weeks. L4 larvae of transgenic *C. elegans* expressing wild-type or ALS mutant G85R SOD1 in all neurons were examined for their movement phenotypes while growing on different

concentrations of NVP-BEP800. The behavioral tests were attempted with both manual observations and computer-assisted software. In the manual test, worms were prodded with a platinum pick on the tail or the nose, and immediately after receiving the touch stimulus, the worms exhibit an escape response and their crawling speed reflects their neuromuscular functional status. Using this assay, we carefully observed whether the drug treatment would improve a movement defect observed on the ALS mutant SOD1-G85R *C. elegans* as compared to the wild-type SOD1 animals. After several trials, we concluded that the drug treatment did not significantly affect the movement behaviors in the mutant SOD1 *C. elegans.*

We also attempted to establish a more objective and sensitive method to use computer software to measure the *C. elegans* movement phenotypes. Over 50 animals in each strain were videotaped, and their movements were analyzed with a commercial tracking and analyzing software, WormLab (MicroBrightfield, Inc). The software automatically tracks worm movements and analyzes multiple parameters including speed, body wavelength, and bending angels. However, our statistical analysis of the software-generated data did not reveal a significant change on the behavior of the mutant SOD1 *C. elegans*. One of the complicating factors that we realized was that only unstimulated movements could be videotaped and these movements were very variable and frequently interrupted by resting moments of the animals. Improvement of the behavior test such as videotaping the animals in swimming could be further investigated.

Aside from the SOD1 transgenic *C. elegans*, we also examined similar transgenic strains that express human TDP-43 in neurons. We did not observed a significant change on the behavior of the TDP-43 worm model either. Together, we conclude that the HSP90 inhibitor NVP-BEP800 does not have a significant effect on the proteotoxicity in *C. elegans* models of ALS. One interpretation of the result is that the drug may have specificity for the mammalian HSP90 and therefore does not inhibit the orthologous target in *C. elegans.*

B. HSP90 inhibitor NVP-BEP800 significantly reduces SOD1 protein aggregation in mammalian cell models of ALS.

To determine whether NVP-BEP800 also has anti-proteotoxic effects on SOD1 aggregation in mammalian systems, we employed a cell-based model of protein aggregation that we developed previously (Wang J et al., 2003). In brief, mammalian cells were transfected with ALS mutant SOD1-G85R and treated with a series of varying concentrations of NVP-BEP800, before being subjected to a biochemical analysis for protein aggregation. The mammalian cells were first extracted in a 200 µl buffer containing 10mM Tris-HCl pH8.0, 100mM NaCl, 1mM EDTA pH8.0, 0.5% NP-40, 50µM iodoacetamine, and protease inhibitor (Sigma) by a Bioruptor ultrasonicator at 4 ^{o}C for 5 min. The lysates were then transferred to an Airfuge ultracentrifuge (Beckman Coulter), and centrifuged at 25 psi (~130,000 g) for 5 min. The supernatant was transferred to clean tubes and saved as the “soluble” supernatant fraction (S). The remaining pellet was resuspended in extraction buffer again and sonciated for 5 min. This resuspended pellet was applied to the Airfuge and centrifuged at 25psi for 5 min. The remaining pellet was transferred to 100µl of resuspension buffer containing 10mM Tris-HCl pH8.0, 100mM NaCl, 1mM EDTA pH8.0, 0.5% NP-40, 0.5% deoxycholic acid, and 2% SDS, and sonicated for 5 min. This fraction was considered as the “insoluble” protein aggregate pellet fraction (P).

We found that NVP-BEP800 has a potent efficacy of reducing mutant SOD1 protein levels in mammalian cells in a concentration-dependent manner (**Figure 1**). In the soluble (S) fraction, the protein levels of wild-type endogenous SOD1 (WT SOD1) were not changed in the presence of the drug. By contrast, the levels of the ALS mutant SOD1-G85R, which migrates faster than the wild-type counterpart on the SDS-PAGE gels, was gradually decreased with increasing concentrations of NVP-BEP800. Furthermore, in the insoluble protein aggregate fraction (P), there was no wild-type SOD1 that could be detected, consistent with the notion that they do not form protein aggregates. In the absence of NVP-BEP800 (first lane), a significant amount of SOD1-G85R protein aggregates could be detected. However, in the presence of NVP-BEP800, with the lowest concentration at 100 nM, the mutant SOD1-G85R was completely cleared. This result clearly demonstrates that NVP-BEP800, as an inhibitor of HSP90, can be used to reduce SOD1 aggregates in mammalian cells.

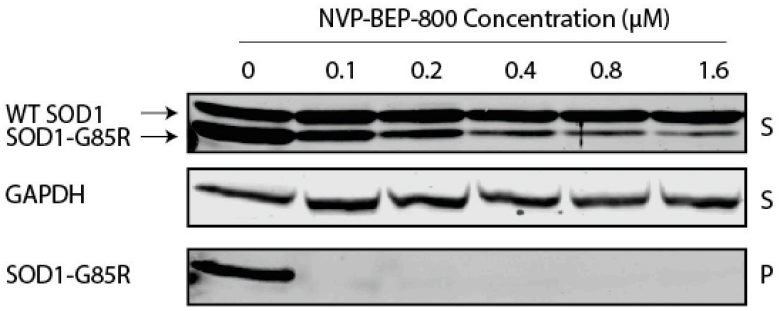

Figure 1. HSP90 inhibitor robustly reduces ALS mutant SOD1-G85R, particularly its aggregated form. Human HEK293 cells were transfected with SOD1-G85R, and the cells were extracted into the soluble fraction (S) and the insoluble aggreate fraction (P).

C. HSP90 inhibitor NVP-BEP800 has a surprising effect of increasing TDP-43 protein levels in mammalian cells.

Next, we repeated the protein aggregation assay as described above for the TDP-43 protein with an ALS mutation M337V. Surprisingly, in contrast to our original hypothesis that a reduction of TDP-43 protein aggregates might occur in response to the treatment of the HSP90 inhibitor, an actual increase in the TDP-43 protein levels was observed (**Figure 2**). In both soluble (S) and insoluble (P) fractions, treatment of NVP-BEP800 significantly increased the proteins levels of TDP-43-M337V in a concentration-dependent manner.

This surprising observation could be a clue to a novel mechanism that was previously unrecognized regarding the regulation of TDP-43. It is relatively well known that the levels of TDP-43 are tightly regulated in many model organisms (Ash et al., 2010; Hanson et al., 2010; Johnson et al., 2008; Kabashi et al., 2009; Li et al., 2010; Liachko et al., 2010; Shan et al., 2010; Stallings et al., 2010; Swarup et al., 2011; Wegorzewska et al., 2009; Wils et al., 2010; Xu et al., 2010; Zhou et al., 2010) (Chiang et al., 2010; Fiesel et al., 2010; Sephton et al., 2010; Wu et al., 2010). Previously, we made an observation of genetic interaction between TDP-43 and HSF-1 orthologs in *C. elegans* (Zhang et al., 2011). However, it is unexpected that an activation of TDP-43 would robustly increase the TDP-43 protein levels. Recently, we discovered that TDP-43 has a function in protein quality control (Zhang et al., 2012). We are speculating that the new observations made here (Figure 2) might suggest that TDP-43 is part of an HSF-1-regulated protein quality control program. Further studies would be required to elucidate the mechanisms and the implications of the findings.

D. Identification of TDP-43's function in the regulation of protein quality control

Consistent with the notion suggested by the results above (Result C) that TDP-43 may

be part of the protein quality control programs, we have recently identified a previously unrecognized function of TDP-43, involving the sensing of proteotoxic stress and direct regulation of protein quality control (Zhang et. al., to be submitted). The main evidence for this finding are as follows.

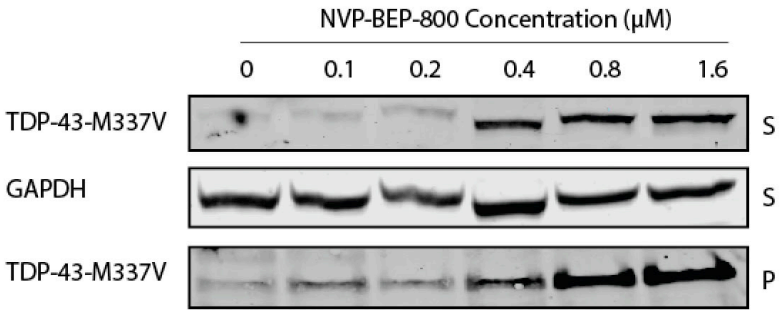

Figure 2. HSP90 inhibition increases the protein level of ALS mutant TDP43-M337V. Human HEK293 cells were transfected with mutant TDP-43, and treated with varying concentration of NVP-BEP-800, before being extracted into the soluble fraction (S) and the insoluble aggreate fraction (P).

We studied the effects of loss or gain of TDP-43 on protein aggregation using a previously established SOD1 solubility assay (as seen in Figure 1). The assay relies on differential detergent extraction to separate insoluble protein aggregates from soluble low-molecular weight monomers and oligomers. The G85R mutant, but not WT SOD1, was found in the insoluble pellet, providing a sensitive reporter for protein aggregation. When TDP-43 was ectopically expressed in HEK293T cells, there was a significant increase in the level of insoluble G85R SOD1 aggregates, with no difference in the soluble level (**Figure 3A**). However, when TDP-43 was knocked down in HEK293T cells, there was a marked reduction in the insoluble aggregates of G85R SOD1, together with a decrease in the level of soluble mutant SOD1 (**Figure 3B**). The WT SOD1 protein level was not changed when TDP-43 was knocked down or overexpressed, suggesting that TDP-43 negatively regulates the turnover of misfolded proteins.

To examine whether the reduction in misfolded and aggregated proteins occurs through increased protein degradation, we measured the global proteasome activity when TDP-43 was knocked down in HEK293 cells. The chymotrypsin-like activity of the proteasome was measured by analyzing a cleavage-dependent luciferin signal on a 20S proteasome model substrate (**Figure 3C**). Knockdown of TDP-43 in HEK293T cells led to a significant increase in the luciferin signal when compared to cells transfected with non-targeting shRNA controls (**Figure 3D**). These results indicate that TDP-43 is a significant modifier of proteasome activity.

Autophagy is another major protein quality control pathway in the clearance of misfolded and aggregated proteins. To determine whether TDP-43 also modifies autophagic activity, we monitored changes in the autophagosome marker LC3 by Western blotting after knockdown of TDP-43. When autophagy is triggered, LC3-I is converted to the lipidated LC3-II and then inserted into the membrane of autophagosomes; therefore, an increase in the amount of LC3-II or the ratio of LC3-II/LC3-I indicates activation of autophagy. Knockdown of TDP-43 in HEK293T cells led to an increase in the amount of LC3-II and in the ratio of LC3-II to LC3-I (Figure 3E), suggesting that autophagy had been

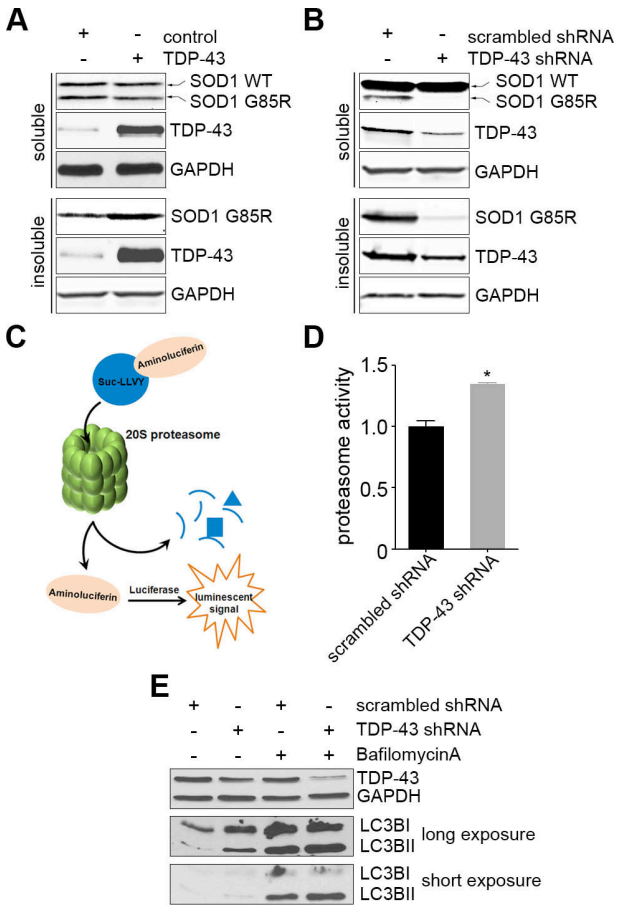

Figure 3. A novel function of TDP-43 in protein quality control in contrast to SOD1.

activated. LC3-II is short-lived and degraded through the fusion of autophagosomes with lysosomes. Therefore, our observed increase in LC3-II levels could have been due to a failure of this fusion event. To confirm that the increase in the level of LC3-II was indeed the result of autophagy activation, we treated the cells with bafilomycin A1, a highly specific inhibitor of vacuolar type H^+-ATPase, which blocks the fusion of autophagosomes to lysosomes. Our results showed that LC3-II levels in TDP-43 knockdown cells were further stabilized after bafilomycin treatment, indicating that the TDP-43-mediated increase in LC3II levels is due to an activation of the autophagic pathway and is not merely a perturbation in autophagosome fusion (**Figure 3E**). Taken together, these results demonstrate that TDP-43 regulates protein quality control by regulating both proteasome- and autophagy-related pathways.

The mechanism undergirding this control of protein quality control is at least partially mediated by TDP-43's regulation of FOXO transcription factors (**Figure 4**, and data not shown). These results provide important insight into the role of TDP-43 in disease pathogenesis. Because of the chronic stress associated with neurodegenerative diseases, the TDP-43 switch is kept in overdrive mode, compromising the protein's capacity to buffer further stress and maintain protein homeostasis. This mechanism also implies that other disease-associated RNA-processing proteins exhibiting similar stress-induced behavior might be coupled to other cellular pathways as part of a coordinated reprogramming of stress responses.

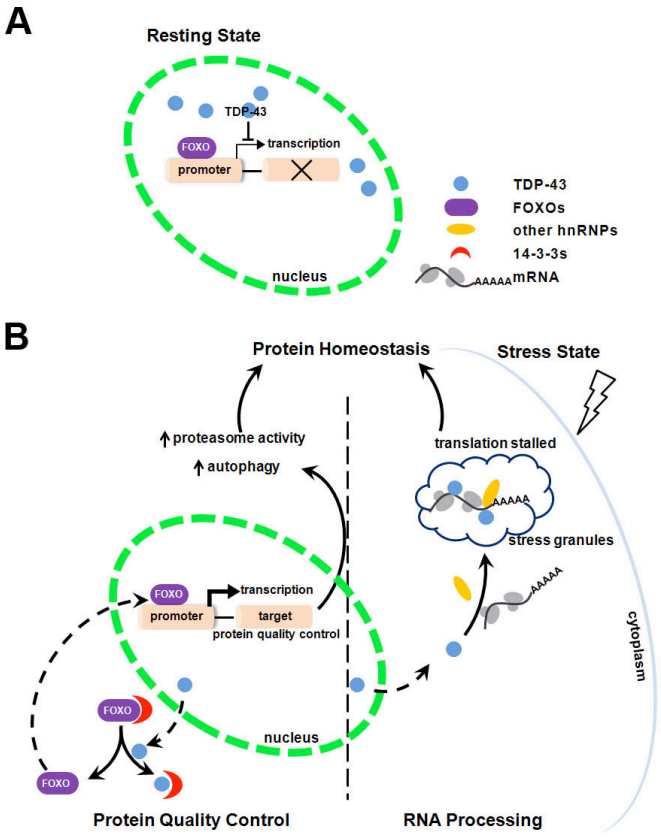

Figure 4. The model for RNA-binding protein TDP-43 functioning in the regulation of protein quality control.

E. On-going progress on testing NVP-BEP800 in mouse models of ALS.

Following up with our studies in *C. elegans* and mammalian cells, we are extending the observations to mouse models. While we were studying the *C. elegans* and mammalian cells, we imported a mutant SOD1 transgenic mouse strain (Jackson Laboratory, stock number 004435) and a transgenic TDP-43 (A315T) mouse model (Jackson Laboratory, stock number 010700). We have established colonies of these strains and had collected initial characterization of these animals.

While establishing and maintaining the transgenic TDP-43 (A315T) mouse model, we noted a phenotype of these mice that has now been confirmed by multiple other labs in the field. Most of the TDP-43 positive mice die between 3-5 months of age of a sudden cause before they exhibit clear neurological signs. Many labs have found that these mice develop an intestinal pathology, which is the likely cause of their death and complicates the studies of the neuromuscular system. It has become a recent consensus of the field that these TDP-43 mice are not suitable for studying modulation of disease onset and progression. Therefore, we have focused on the mouse experiments by using the well-characterized SOD1 mouse models.

We have been breeding the mutant SOD1-G93A mouse strain. The strain has small litters, and females are infertile. As a result, the breeding has been time-consuming. The majority of the SOD1 mice were used in the work for Task 3 in Aim 2. We are now in the process of conducting a pilot study on the effect of NVP-BEP800 on the SOD1 motor behaviors. In our colony, these mice develop clear neurological signs only after 5-6 months. We have been continuing the breeding, which takes longer than we expected to produce the same number of offspring. Otherwise this task is in good progress as planned.

Future work:

In this aim, we made a novel and potentially important discovery that the HSP90 inhibitor elicits a surprising increase in the levels of TDP-43 proteins. Our results suggest that an unknown regulation exists between HSP90/HSF-1 and TDP-43. This observation is particularly intriguing in light of our recent finding that TDP-43 itself regulates protein quality control programs. Because TDP-43 is the most common pathological inclusion marker in sporadic and familial ALS, further understanding of the role of TDP-43 in protein quality control will be critical for the design of wide-spectrum anti-proteotoxicity therapies. We have completed the work in **Task 1** and are focusing on the work in Task 2. In **Task 2**, we will build on our preliminary evidence of reduced mutant SOD1 protein aggregation in cell-based models (Figure 1) and examine how the HSP90 inhibitor NVP-BEP800 might affect disease in SOD1 mouse models. Additionally, we will follow up our findings of HSP90 inhibitor positively regulate TDP-43 levels (Figure 2) and study the novel mechanism of regulation of protein quality control by TDP-43 (Figure 3-4). We will study this regulation both in cell-based models to dissect the molecular mechanisms at both transcriptional and translational levels. This will be innovative research for the field. The mechanisms learnt through this research could reveal new targets in the pathway that would allow us control TDP-43 proteinopathy and related proteotoxicity.

Aim 2: To determine whether SUNS, a newly identified pathway suppressing SOD1/TDP-43 toxicity, offers novel targets for treatment in mouse models of ALS.

Overview:

In this aim, we focus on a pathway that strongly suppresses proteotoxicity-related neurodegeneration, the ***s****pr-5*/LSD1 and ***u****fd-2*/UBE4B-dependent **n**eurodegeneration **s**uppressor (SUNS) pathway. In this aim, we have been developing an *in vivo* approach to knock down UBE4B (**Task 3**). Although we have successfully engineered AAV-shRNA vectors for UBE4B and demonstrated effective knockdown in mammalian cells, the knockdown effects in mouse spinal cords have not been optimal (Results A-E below). We will continue optimizing the *in vivo* delivery approach AAV vectors, while testing easily administered small molecule drugs on novel targets in the SUNS pathway.

UBE4B and LSD1 act synergistically in the SUNS pathway, but their shared downstream effectors remain unknown. We have systematically profiled the changes in the transcriptome following individual or simultaneous inactivation of UBE4B and LSD1 (Fig. 9). Remarkably, following leads concerning differentially regulated genes, we noted a previously unrecognized activity of the p53 transcription factor that enhances the degradation of misfolded proteins. Our preliminary studies suggest that p53 is likely a critical effector downstream of UBE4B and LSD1 that regulates clearance of misfolded proteins. We have preliminary evidence that small molecules that activate p53 reduce the aggregation of mutant SOD1. We plan to examine the

effects of p53 small molecule activators on the disease phenotypes in SOD1 mouse models of ALS (**Task 4**). Our recognition of this activity of p53 acting in the SUNS pathway represents an exciting discovery of a potentially novel protein degradation pathway and opens new avenues for developing novel targets for "wide-spectrum" anti-proteotoxicity therapeutic strategy.

Task 3: Examine the effect of the SUNS pathway on ALS mouse models by establishing an *in vivo* approach to knock down UBE4B.

Task 4: Determine whether p53 is a downstream effector of UBE4B and LSD1 and its activation can delay disease onset and progression in ALS mouse models.

Results and Findings:

Task 3:

A. The cloning of AAV9-UBE4BshRNA and viral production.

In order to develop an *in vivo* approach to knock down UBE4B, we explored the small hairpin RNA (shRNA) mediated gene interference. We collaborated with Dr. Brian Kasper, an expert on viral gene delivery to the central nervous system, and adopted his AAV9 system based on recently established advantages (Foust et al., 2009). We first cloned an shRNA sequence targeting the mouse Ube4B under a U6 promoter into the AAV9 viral construct (**Figure 5**), using the INFUSION Clone technique. Oligo primers were used to amplify the U6-UBE4B-shRNA sequence on the "Ube4B.71.RFP.C.RS" plasmid. Then the AAV9 plasmid was cut at KpnI site and ligated with the insert. The scAAV plasmid can co-express GFP under the control of the CMV promoter. The final cloning product "shUBE4B.71.AAV.GFP" was verified by sequencing.

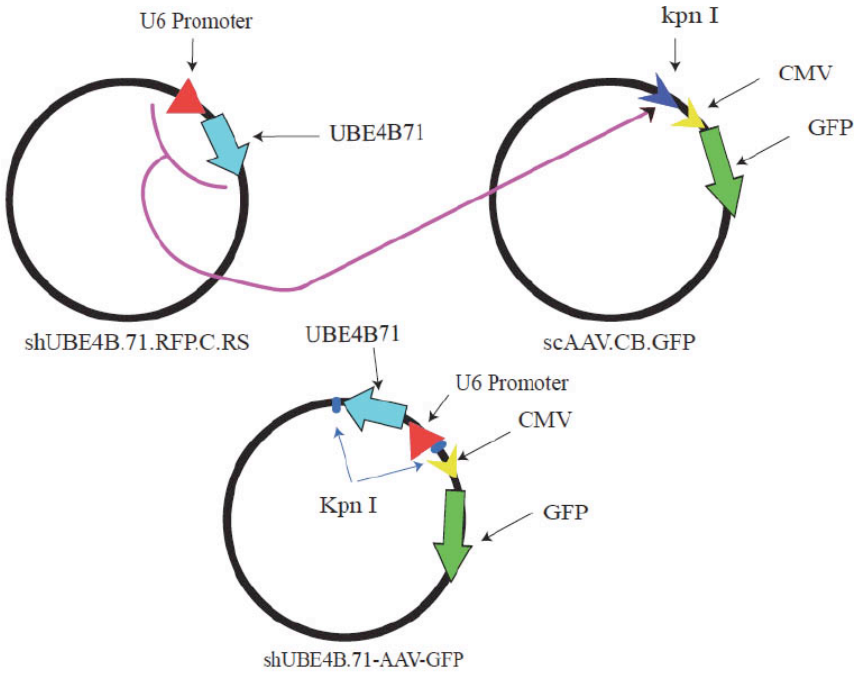

Figure 5. The scheme of cloning AAV9-UBE4BshRNA.

We tried viral production and packaging first in the lab, but the yield didn't reach the desired titer. Therefore we outsourced the production of the virus to a company, Virovek. The tittering of the viral product was determined to be at 2.3 X 10^{13} particles/ml. These viruses were used for the further studies.

B. The knockdown of Ube4B by the AAV9-UBE4BshRNA construct in mammalian cells.

To verify the knockdown efficacy of the UBE4BshRNA construct, we first transfected the viral plasmid using Lipofectamine into mouse 3T3 cells. After transfection, the cells were harvested and the level of endogenous mouse Ube4B protein was assessed by Western

blotting. Compared to a control shRNA, the UBE4BshRNA construct significantly knocked down the Ube4B protein (**Figure 6**). This result indicates that the specific shRNA sequence is effective in knocking down the Ube4B target.

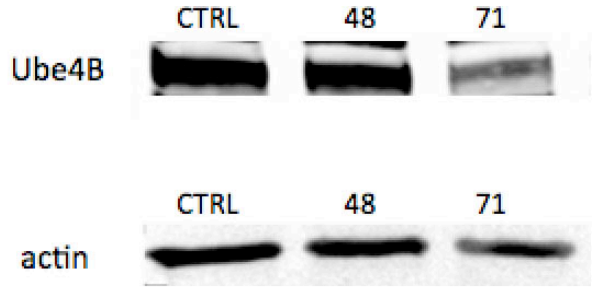

Figure 6. The knockdown of Ube4B by the shRNA construct in mouse 3T3 cells. Western blots using an antibody for Ube4B is shown after the cells are treated with anti-Ube4B shRNA (71) or a control (48). Actin is shown as a loading control.

Next, we tested the knockdown efficacy of the AAV9 virus produced by the same construct. The virus was used to infect mouse 3T3 cells, and the Ube4B protein was assessed. According to Virovek, AAV9 has limited infectivity in cultured cells as compared to in animal tissues. Our observations were in agreement with this assessment. About 1 X 10^{10} viral particles were used to infect 5 X 10^{4} cells, on day 3 only about 10-20% of the cells showed green fluorescence, which was the indication that the viral DNA was being transcribed. Of the cells that expressed GFP, there was no indication of decrease in Ube4B protein level based on the intensity of the immunofluorescent staining. It is possible that the immunofluorescent signals are not sufficiently quantitatively, or the viral DNA was not transcribed as abundantly as the transfected plasmids. Nonetheless, we proceeded forward to test the virus in mice.

C. The *in vivo* delivery of AAV9-UBE4BshRNA virus to spinal cords of wild-type and SOD1 transgenic mice.

A major challenge in studying viral gene therapy for motor neuron diseases is the delivery of the virus to spinal cords. Initially, we tried a few delivery approaches including injecting into cerebral lateral in neonatal mice or injecting into tail vein of adult mice. Neither of the approaches was reliable for us to deliver the virus. Finally, we collaborated with Dr. David Borchelt in University of Florida, who recently developed a method of injecting virus intraparenchymally directly into spinal cords of neonatal mice, following approved anesthesia (hypothermia), that had been more reliable than other approaches. The injections were done in a sterilized hood with a 33 gauge, 1.0 inch, 12 degree point, sterile needle connected to a 10ul Luer-hub Hamilton syringe. The injection mixture was made from the original Virovek viral mix with a green food dye. 2uL of the mixture was injected to the P0/P1 pup according the protocol. The injected pups all showed a clear green line across the spine, ranging from the neck area to the injection site. These pups were put back to cages after injections.

D. Examination of successful of AAV9-UBE4BshRNA infection in spinal cords.

At about twenty-one to twenty-five days post spinal injection mark, injected mice were anesthetized by isofurane, then perfused 4% paraformaldehyde. Spinal column was dissected and fixed. After extraction of the spinal cord by laminectomy, spinal cords were further fixed and impregnated. Finally, three sections of the spinal cords (cervical, thoracic, lumbar), each with length of about 3mm, were embedded in OCT (Tissue-Tek, Torrance, CA) and frozen with ethanol and dry ice. Tissue sections of 20 μm were cut using a cryostat. These slides were immunostained for Ube4B and NeuN, and observed under a Zeiss Apotome microscope.

We found that AAV9-UBE4B71 was successfully introduced into the spinal cord. The GFP reporter showed that AAV9-UBE4B71 infected all cell types in the spinal cord. However,

the infectivity did not result in an obvious reduction in Ube4B immunofluorescence
(**Figure 7**). To our knowledge, this is the first study to stain Ube4B in tissue, and there is no other reported Ube4B immunostaining in the literature. Since Ube4B is an under-studied protein, better antibodies would be needed for improved studies in the future.

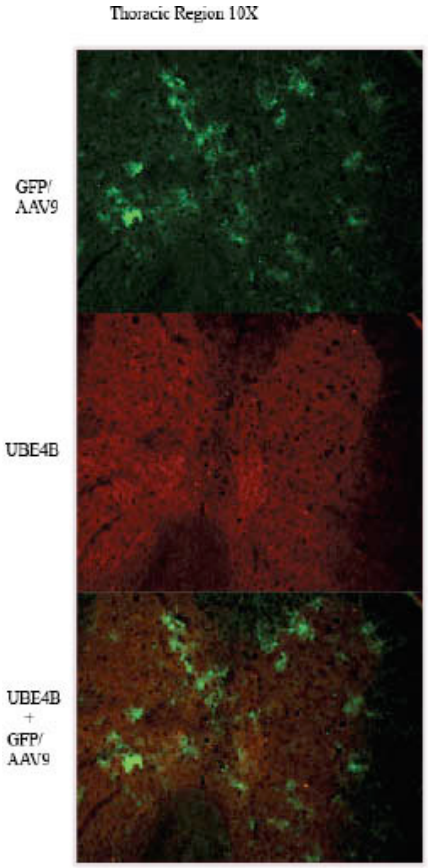

Figure 7. Mouse spinal cord expression patterns of UBE4B protein and the GFP marker from AAV9-UBE4B71 injected.

E. Alternative method to knock down UBE4B via antisense oligonucleotides

Since the efficacy of using the shRNA approach to knock down UBE4B requires further proof and development of new reagents such as better antibodies for UBE4B immunostaining, we opt to explore alternative method to knock down the mouse Ube4B gene. We explored the approach to knock down the gene by using chemically modified antisense oligonucleotides (ASO). ASOs have been used for decades to silence genes, and constantly improving technologies have made it an effective approach in both animal models and clinical trials, especially for delivery to the central nervous system for combating neurodegenerative disease. Among the commercially available options, we chose (locked nucleic acid) LNA-Gapmers from Exiqon, which are a special design of a sandwich of LNA and DNA that is shown to have potent and highly efficient inhibition of mRNAs. We designed five different LNA-Gapmers targeting the Ube4b gene. We then performed a screening of these candidate ASOs in tissue cultures to select the most effective design, and found that they have varying degree of knocking down the endogenous Ube4B protein levels (**Figure 8**). Therefore, we have identified an alternative method to successfully knock down the Ube4B gene.

Task 4:

F. Identification of p53 as a critical effector downstream of UBE4B and LSD1 in the SUNS pathway.

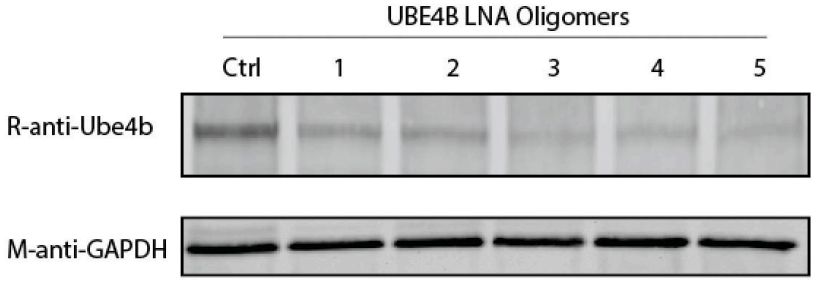

Figure 8. The knockdown of mouse UBe4B gene using ASO LNA GAPmerRs. Mouse 3T3/MEF cells were transfected with different ASOs (1-5) with Lipofectamine LTX (Life Technologies). 50 picamoles of each ASO were transfected to 3 X 10^4 cells/ml in a 12-well plate. 48 h later, cell lysates were analyzed by Western blots with a rabbit anti-Ube4b antibody and a mouse anti-GAPDH control.

It remains unknown what are the targets/effectors of UBE4B and LSD1 in the anti-proteotoxicity pathway. We speculate that the ubiquitin ligase UBE4B and the lysine-specific demethylase LSD1 regulate shared downstream effectors involved in protein quality control, and that inhibition of these two enzymes activates the specific protein quality control pathway to suppress proteotoxicity-related neurodegeneration. To identify the shared targets of UBE4B and LSD1, we recently performed a systematic transcriptional profiling analysis of the UBE4B/LSD1-mediated pathway. This analysis has uncovered a novel protein quality control pathway that we are excited to pursue further.

To determine the changes in gene expression profiles that occur upon the inactivation of UBE4B and LSD1, we compared whole transcriptomes of the human cell model expressing ALS-linked mutant SOD1 under different conditions in which either LSD1 or UBE4B is knocked down, alone or in combination. Mutant SOD1 was included as a reporter for the activation of the anti-proteotoxicity pathway. We used the Affymetrix Gene 1.0 array platforms, and included biological triplicates in each treatment. The cDNA library construction and microarray analysis was conducted at the Johns Hopkins Core Facility. In double vs. control knockdown condition, 1860 transcripts are affected greater than 1.2-fold and with p value ≤ 0.05 (out of total 22148 annotated transcripts on Affymetrix human GENE 1.0ST array chip).

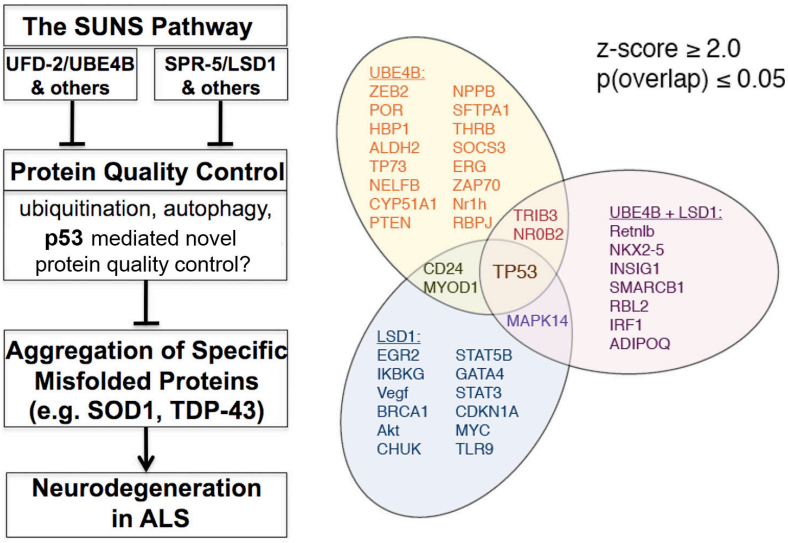

Fig. 9 Novel targets of reprogramming protein quality control. (Left) UBE4B and LSD1 are founding members of the novel SUNS pathway, which antagonizes the aggregation of specific protein substrates and suppressing neurodegeneration. p53 emerges as a novel downstream effector of the SUNS pathway. (Right) Transcriptome profiling of the SUNS pathway demonstrates that p53 is a common effector of both UBE4B and LSD1.

The most intriguing observation from the microarray expression study is made not through analyzing individual down or up-regulated genes but through analyzing pathways controlled by upstream regulators. Using Ingenuity Pathway Analysis, we found that p53, as a transcription factor, is the only transcription factor strongly activated in double UBE4B-LSD1 knockdown samples (**Figure 9**).

Another important preliminary finding is our observation that p53 can directly suppress the aggregation of misfolded proteins such as G85R SOD1. We demonstrated this suppression by using an existing drug that is a known activator of p53, tenovin-1. Tenovin-1 is a SIRT1/2 deacetylase inhibitor that increases the acetylation, stability, and activity of K382 of p53 (Lain et al., 2008). We transfected the G85R SOD1 reporter into HEK293T cells and treated the cells with various concentrations of tenovin-1. Remarkably, at 0.8 μM the inhibitor was able to significantly reduce the production of aggregated forms of G85R SOD1 (**Figure 10**). These experiments have established thus far that p53 is a downstream effector of UBE4B and LSD1 with a previously unrecognized role in activating protein quality control.

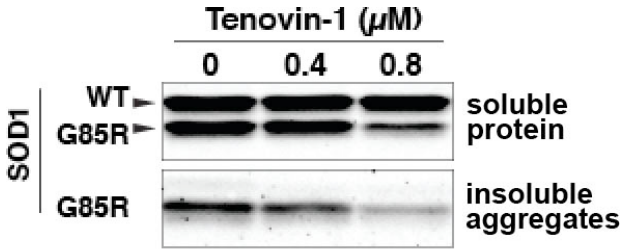

Fig. 10 p53 activator Tenovin-1 promotes clearance of misfolded and aggregated mutant SOD1 (G85R) protein.

Future work:

In this aim, we will continue optimizing the *in vivo* approach to knock down UBE4B through shRNAs in spinal cords (**Task 3**). Toward this end, we will deliver the shRNAs through injection in neonatal mice with ACUC approved procedures. Additionally, to verify the proteins levels of UBE4B in spinal cord neurons, we will optimize the immunohistochemistry staining protocol in the mouse central nervous system. Furthermore, to evaluate the combined effects of UBE4B and LSD1 in the SUNS pathway, we will examine the effects of modulating the shared

downstream effector, p53, as a potential target (**Task 4**). Following up with our preliminary data that p53 activator, Tenovin-1, suppresses aggregation of mutant SOD1 proteins, we will test how this drug may affect the disease onset and progression in the SOD1 mutant transgenic mouse model with an ACUC approved procedure.

Key Research Accomplishments

- Revealed that the HSP90 inhibitor NVP-BEP800 is specific to mammalian targets but not the *C. elegans* counterparts.
- Demonstrated that the inhibitor NVP-BEP800 has a potent effect of reducing misfolded mutant SOD1 in mammalian cells.
- Observed a novel finding that the inhibitor NVP-BEP800 has a surprising effect of significant increase TDP-43 protein levels.
- Confirmed behavioral phenotypes in the mouse models of SOD1 and TDP-43 related ALS.
- Cloned and characterized a viral shRNA construct that knocked down the mouse Ube4B target in the cell.
- Successfully demonstrated an *in vivo* approach to deliver the virus to mouse spinal cords via intraparenchymal injection. This overall a key technical hurdle in the animal studies of the gene therapy approach.
- Demonstrated an alternative approach to knock down the target gens of UBE4B via antisense oligos.
- Identification of a novel p53-mediated protein quality control program downstream of the UBE4B-LSD1 anti-proteotoxicity pathway.
- Demonstration that a p53-activating drug has the anti-proteotoxicity activity.

Reportable Outcomes

1. Paper to be submitted for publication: “RNA-processing Protein TDP-43 functions as a Regulator of Protein Quality Control”. Zhang, T. et. al. (2014)
2. Paper to be submitted for publication: “UBE4B and LSD1 as novel suppressors of protein aggregation”. Periz, G. et. al. (2014)
3. The master thesis “AAV Mediated Knockdown of Gene Targets in ALS Mouse Models” by Ms. Qingwen Kawaji based on her work on this project was approved by the Johns Hopkins Bloomberg School of Public Health in July, 2013.
4. A Master of Science degree was awarded to Ms. Qingwen Kawaji for her excellent research

work on this project in the summer of 2013.

5. Ms. Sarah Kavianpour, who worked on this project and was supported by this award, was able to secure a full-time Research Technologist position in the Johns Hopkins School of Medicine based on her experience on animal tissue histology.

6. We developed a potential therapeutic agent by generating and characterizing the AAV9 vector expressing an shRNA that specifically targets UBE4B.

7. We also developed research agents that have therapeutic potentials by screening and characterizing antisense oligos that specifically target UBE4B.

Conclusion

In summary, we have made significant progress in the first year of the project. In Aim 1, we have completed the investigation using *C. elegans* and mammalian cells that evaluated for the first time the effects of an HSP90 inhibitor as a candidate anti-proteotoxicity agent on ALS mutant proteins. We discovered that the HSP90 inhibitor was a potent inhibitor of SOD1 protein aggregation, and surprisingly, could significantly up-regulate the TDP-43 proteins levels. This finding indicates that SOD1 and TDP-43 have different mechanisms of regulations. The results suggest that inhibition of HSP90 is a promising approach to suppress aggregation of SOD1 but not TDP-43. Importantly, the results also suggest that there exists a novel mechanism for HSP90/HSF1 to regulate TDP-43 in mammalian cells. In Aim 2, we successfully developed a viral approach to deliver shRNA to knock down the target gene UBE4B. We have achieved the specificity of the knockdown and overcome the delivery approach to the mouse spinal cords. As an alternative approach, antisense oligos were shown to effectively knock down the target gene UBE4B in mammalian cells. Together, our findings provide important new knowledge regarding the basic mechanism of ALS-related protein aggregation and demonstrate significant progress towards evaluating candidate targets in mammalian models.

In light of these new findings, in the second year of the project, we will continue the direction of studying ALS proteotoxicity as planned with the following recommended changes. In Aim 1, we had originally planned to test the HSP90 inhibitor drug on a large number of ALS mice including the TDP-43 mice. Our finding that the inhibitor drug has differential effects on the aggregation of SOD1 and TDP-43 will complicate the results and interpretation on the mice. Since the finding suggests a novel mechanism for HSP90/HSF1 to regulate TDP-43, and the TDP-43 proteinopathy is the most common hallmark for sporadic and familial ALS, e propose to scale down the original plan of testing a large number of ALS mice and refocus on the elucidation of novel mechanism of regulation of TDP-43 by HSP90/HSF-1. We will first investigate the detailed mechanisms using cell models of TDP-43 proteinopathy and extend any positive findings to mice. In Aim 2, given the results that the UBE4B shRNA has sufficient efficacy to knock down the target gene when highly expressed in cell models but has lower efficacy in spinal cord neurons, we would like to recommend limited changes that would allow us to not be fully dependent on the shRNA approach but explore alternative approaches such as antisense oligos and new targets in the UBE4B pathways. We propose to systematically study the gene expression profiles induced by the knockdown of UBE4B and define new downstream targets that could also be utilized to develop potential therapeutic agents.

"Scientific Product" in this report: The work revealed that HSP90 inhibitor was a potent inhibitor of SOD1 protein aggregation and therefore a potential therapeutic agent for this form of ALS. A novel mechanism was discovered regarding a regulation of HSP90/HSF1 on TDP-43, and further studies could lead to important insights into this common disease factor of ALS. UBE4B as a candidate therapeutic target could be specifically targeted and knocked down via shRNA, which could be successfully delivered to spinal cords by AAV virus. P53 was identified as a promising downstream effector in the SUNS pathway that could be targeted for disease interventions.

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Appendices

Appendix A: Revised Statement of Work (SOW).

Appendix B: IACUC-approved form of changes in procedure.

Statement of Work (SOW)

revised February 10, 2014

Task 1: Test the therapeutic effects of the HSP90 inhibitors on SOD1/TDP-43-induced toxicity in both cell and mouse models of amyotrophic lateral sclerosis (ALS).

a) Test the neuroprotection potential of the HSP90 inhibitor NVP-BEP800 in *C. elegans* models of SOD1– and TDP-43–induced neurodegeneration. We already have established these *C. elegans* models and have successfully used them to evaluate the neuroprotecive potential of several agents. We will use these same models to examine the effect of several NVP-BEP800 concentrations on motility and behavior in *C. elegans* as indicators of their neuromuscular function. (Timeframe: Months 1-6).

b) Evaluate the neuroprotective properties of NVP-BEP800 in mammalian cell-based systems. The degree of TDP-43 aggregation in mammalian cell model will be examined in the absence or in presence of several different concentrations of NVP-BEP800. Methods for analyzing and quantitating protein aggregation *in vivo* are well established in our lab, and the effects of the inhibitor can be assessed quickly. The potential effect of NVP-BEP800 in reducing TDP-43-induced toxicity will be examined by both survival assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and morphological assessment of the neuronal processes (Timeframe: Months 1-6).

Task 2: Examine regulations between protein quality control systems and ALS proteins such as SOD1 and TDP-43. The putative protective effects of NVP-BEP800 in cell-based models will be evaluated in mutant SOD1 mouse models. Transgenic SOD1 (G93A) mice start to show symptoms of ALS-like disease at ~3 months of age. The treatment will begin at 30 days of age, using at least 20 age- and gender-matched animals in the experimental group and the control. Disease onset and progression will be monitored by assessing locomotor performance and survival. Additionally, we will study the novel mechanisms of regulation of protein quality control by TDP-43 in mammalian cells (see justifications provided by new results shown in Figures 2-4). (Timeframe: Months 2-24).

Task 3: Examine the mechanisms of the SUNS pathway and the feasibility of activating this pathway by knocking down UBE4B in mouse models. We have discovered that removal of UBE4B and KDM1A in the SUNS pathway synergistically inhibits SOD1- and TDP-43-induced proteotoxicity and suppresses SOD1 and TDP-43 aggregation in *C. elegans* and cell-based models. Since downregulation of UBE4B alone significantly suppressed protein aggregation, we will first determine whether UBE4B can be successfully knocked down in vivo in mouse models. Furthermore, we will study the mechanisms of the SUNS pathway in order to identify downstream effectors and potential new targets for interventions.

3a) *In vivo* knockdown of UBE4B using AAV viral delivery of shRNA expression constructs. In our previous studies, we validated a set of shRNAs that are effective in knocking down UBE4B in cell-based systems. We will use the same shRNAs to produce infective AAV virus that can deliver shRNA to the adult mouse central nervous system including spinal cords. The SOD1 mouse modes will be used as recipients of either control (ineffective) shRNA or shRNA against UBE4B. AAV virus will be injected into the mouse central nervous system, and disease onset

and progression will be monitored by measuring body weight, locomotor performance, and survival. Histological analyses of the control and UBE4B-knockdown spinal cords will be performed in 120-day-old mice. Moreover, we will explore the use of antisense oligonucleotides as an alternative strategy for UBE4B knockdown. The efficacy of this approached will be tested in cell-based models, since the tests in mice will require time and resources beyond the scope of this project. (Timeframe: Months 2-24).

3b). It remains unknown what are the targets/effectors of UBE4B and KDM1A in the anti-proteotoxicity pathway. Identification of such shared effectors downstream of UBE4B and KDM1A would provide single targets for possible interventions. To identify the shared targets of UBE4B and KDM1A, we will perform a systematic transcriptional profiling analysis of the UBE4B/ KDM1A-mediated pathway. The analysis of the most promising effectors will be carried out in the context of the anti-proteotoxic SUNS pathway in order to identify novel wide-spectrum targets. (Timeframe: Months 7-24).

Task 4: Determine whether targeting the common effectors in the SUNS pathway downstream of UBE4B and KDM1A has an improved effect in alleviating disease onset and progression in ALS mouse models (see justifications provided by new results shown in Figures 9-10). Our preliminary results suggest that p53 is a promising downstream effector mediating the action of UBE4B and KDM1A in their synergistic effect of alleviating proteotoxicity and aggregation. Unlike UBE4B for which no small molecule inhibitor or effective knockdown is exiting, p53 has well-established drugs to study its activities. We will test this hypothesis that activation of p53 via drugs mediates the action of the SUNS pathway and protects against proteotoxicity-mediated neurodegeneration in SOD1 mouse models. (Timeframe: Months 16-24).

JOHNS HOPKINS
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Animal Care and Use Committee

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To: Dr. Jiou Wang
Department of Biochemistry - Sch Public Health

From: Nancy A. Ator, Ph.D.
Chair, Animal Care and Use Committee

Date: 02/11/2014

Subject: Amendment Approval Memo

On 02/11/2014, the Johns Hopkins University Animal Care and Use Committee (ACUC) approved the following [Procedures/ amendment to your research protocol. A copy of the approved amendment is attached.

Protocol Number: MO12H231

Title: The mechanisms of neurodegenerative diseases REPLACES MO09H303

Expiration Date: 06/29/2014

Additional modifications to this protocol can be requested by submitting the appropriate amendment form (i.e., Change in Animal Number, Change in Personnel, or Change in Procedures) to the ACUC office for review and approval. Copies of all current forms can be found on our website: www.jhu.edu/animalcare.

For guidance on protocol modifications that require amendments, please refer to the reverse side of this letter. If the locations for outside housing or procedures change, please submit a Change in Location Form, also available on the website.

Johns Hopkins University Animal Care and Use Committee

CHANGE IN PROCEDURE(S) OR ANIMAL NUMBERS AMENDMENT REQUEST FORM

Release date: 12/08

Below for ACUC Use	
Date Received:	2/5/14
Expiration Date:	6/29/14
☑ Logged	☑ Database

Protocol Number: MO12H231

Protocol Title: The Mechanisms of Neurodegenerative Diseases

Principal Investigator: Jiou Wang

Department: Biochemistry and Mol. Bio. School: BSPH

Building: BSPH Room: E8628 Campus: East Baltimore

Office Phone: 502-0927 Fax: 955-2926 E-mail: jiouw@jhmi.edu

If this request is being faxed or emailed (with an electronic signature) to the ACUC Office, an original is not needed.

Please indicate which changes you are requesting by an **X** next to each category below. Describe the change(s) and reasons on page 2 of this form. Please return a **signed copy** of this form to the ACUC Office, Reed Hall, room B122 or fax to 443-287-3747 (7-3747).

To add new personnel or change the PI, please complete the **Change in Personnel Amendment Request Form** or **Change in PI Amendment Request Form.**
To change a location for animal use complete the **Change in Location Form.**
All forms are available on the web at www.jhu.edu/animalcare/forms1.html.

_____ **Modify anesthetic or analgesic agents:** *State the name of the agent, dose or dose range, route of administration and frequency range for any drug to be added. Previously approved agents will remain on the protocol. If you need to withhold analgesia, indicate the reasons why and see "Modify Pain Category" below to see if it applies.*

_____ **Modify Euthanasia:** *Describe any changes in the method of euthanasia (be sure proposed method is in compliance with the 2007 AVMA Guideline on Euthanasia, which can be viewed at www.avma.org/resources/euthanasia.pdf)*

__X__ **Modify Procedures:** *Provide a complete description and rationale for the proposed experimental changes. Indicate if they will change the degree of invasiveness of a procedure or discomfort to the animal. (i.e., the withholding of analgesics; change from non-survival to survival surgery; change in number, duration, or frequency of procedures performed on the animal, etc.). See "Modify Pain Category" below to determine if it applies.*

_____ **Modify Surgical Procedures:** *Describe any changes to approved surgical procedures.*

_____ **Modify Radiation; or Radioactive, Infectious or Biohazardous Agent:** *Provide rationale for adding this new agent, list all necessary safety precautions, and describe any modifications you plan to make to your currently-approved procedures. Attach pertinent approval letter or copy of application from Health, Safety & Environment as appropriate).*

_____ **Modify Animal Numbers:** *Indicate the number of **additional** animals you are requesting that will fall under each pain category in the chart below. Provide a justification for the change in animal numbers. Each animal should be categorized only once. If adding animals or procedures to category D or E for the first time, please see "Modify Pain Category" below.*

Number Requested	*Pain Category*
	B Breeders
	C No pain or distress
	D Alleviated Pain or distress
	E Unalleviated Pain or distress

_____ **Modify Pain Category:** *Please describe the changes that will affect the pain category. If adding animals or procedures to category D or E for the first time, please include a description of what alternatives to procedures*

that may cause more than momentary or slight pain or distress have been considered and why no alternative was selected. See questions 17b-e on the full protocol form for the information that should be included with respect to category D or E procedures.

_____ **Add Satellite Housing:** *Include Satellite Housing amendment with this form*

_____ **Other:** *describe on page 2.*

CHANGE IN PROCEDURE(S) AMENDMENT REQUEST FORM

Describe the requested change(s) following the guidelines for the specific modification as per page 1 of the form (attach additional pages as necessary).

Procedure 1: Administration of small molecule compounds to mice.

Experimental design: *We previously identified a pathway that strongly suppresses proteotoxicity-related neurodegeneration, the SUNS pathway. We have found that p53 is a critical effector in this anti-proteotoxicity pathway, and that p53 can directly suppress the aggregation of misfolded proteins such as ALS-linked mutant SOD1 G85R. We demonstrated this suppression by using an existing drug that is a known activator of p53, tenovin-1 in cell-based models. Tenovin-1 is a SIRT1/2 deacetylase inhibitor that increases the acetylation, stability, and activity of K382 of p53. In the present study, we will test the effects of Tenovin-1, which is well tolerated in mice and has an established pharmacokinetic profile, on the SOD1 mouse model of ALS.*

Procedures: *Based on the Tenovin-1 pharmacokinetic profile and tumor-suppression data in mice (Lain, S. et al.), we will treat mice with the Tenovin-1 at 50 mg/kg every 3-4 days for 30 days or longer. The drug will be administered as 70% cyclodextrin solution at 20ml/kg through intraperitoneal injections. A likely phenotype of the ALS mice used in this study is limb paralysis and loss of motility. We will compare the drug-adminstered group to the controls by monitoring their motor behaviors. Animals will be judged as reaching an endpoint of such a condition if either unable to feed or drink on their own or if failing to recover an upright position 30 seconds after being turned over, at which point they will be euthanized. These proposed procedures will not change the degree of invasiveness or discomfort to the animal.*

Reference:

Lain, S., Hollick, J.J., Campbell, J., Staples, O.D., Higgins, M., Aoubala, M., McCarthy, A., Appleyard, V., Murray, K.E., Baker, L., et al. (2008). Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. Cancer Cell 13, 454–463.

I understand that these changes must not be implemented until I receive approval for the changes from the Animal Care and Use Committee.

PI Signature: _____ Date: 2-5-2014

IACUC Chair's Signature: _____ Date: 2/11/14